AN EXPERIMENTAL BASIS FOR ESTIMATING THE VIRULENCE OF TUBERCLE BACILLI

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(With 4 Figures in the Text)

The pathogenicity to animals of human and bovine strains of *Mycobacterium tuberculosis* has often been studied and has led, in the past, mainly to the identification of a number of attenuated strains (Griffith, 1919; Jensen & Frimodt-Moller, 1936). Such work has relied mainly upon the study of qualitative changes in the lesions in guinea-pigs and rabbits. Possibly owing to difficulties in arranging these animals in balanced groups, no satisfactory quantitative standards of virulence have yet been defined, and there is therefore very little information as to whether fine variations in virulence ever occur.

A quantitative study of this question therefore demands rigid experimental conditions, especially with regard to allowances for variability of response in the host, and accurate adjustment of inocula. Viable counts of the inocula were used by Schwabacher & Wilson (1937) to establish the conditions under which mice could be infected with tubercle bacilli. Since then, detailed studies have been made of the doses required (Pierce, Dubos & Middlebrook, 1947), pathogenesis (Raleigh & Youmans, 1948), stabilization (Martin, 1946; Hart & Rees, 1950), effect of diet (Sengupta & Howie, 1949), and other factors relevant to the experimental infection of mice. Stewart (1950) described the graduated responses, and differences in the lesions, which were observed when mice were infected intravenously with different strains and different doses of tubercle bacilli. In our present approach to the problem of investigating differences in the virulence of human tubercle bacilli, we have studied a more sensitive graduated-response effect, obtained when mice are infected intracerebrally with cultures at the peak of growth in synthetic liquid medium, according to the technique of Dubos and his colleagues (1947).

METHODS

(1) *Preparation of inocula.* Strains of *M. tuberculosis* were maintained by serial subcultures at intervals of 2–3 weeks on Löwenstein-Jensen slopes. For the tests about 5 mg. moist weight of bacilli were transferred from slopes to a range of 10 ml. quantities of a synthetic medium containing Tween 80 and bovine albumin fraction V (Dubos & Middlebrook, 1947). After 11–12 days' growth, the cultures were gently shaken, coarse clumps allowed to settle and the more or less uniformly turbid supernatant liquids withdrawn. If clumps visible to the naked eye

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were still present in any one culture, all the cultures to be used were shaken mechanically with sterile glass beads for 15 min., and again inspected. One culture from the range of the standard strain H 37 Rv was then selected, approximately equivalent in optical density to a neutral grey screen (Ilford O.25 D) at 100 % light transmission. The other cultures were matched turbidimetrically against this standard, adjustment being made by simple dilution, or in the case of cultures showing less turbidity, by preliminary centrifugation followed by dilution. As a further guide to matching, the packed cell volumes of test and standard cultures were estimated by centrifugation at high speed in tubes with a reservoir and graduated capillary stem. Aliquot portions were then removed from each adjusted culture; one portion was diluted in saline serially to 10^{-4} (sometimes to 10^{-3} or 10^{-5}) for direct colony counts, usually in two replicates of six drops of diluted culture per plate; the other identical portion was used for inoculating the mice within as short a time as possible and at least within 1 hr. from the beginning of dilution for the count.

The procedure detailed above was devised to provide approximately equal viable inocula, with a measure of any differences, whereby various test strains could be compared in effect with the standard strain H 37 Rv. In some experiments the procedure was varied to test the effect of ranges of different sized inocula as well as of suitably adjusted younger or older cultures.

Cultures prepared and adjusted as described above appeared uniformly turbid to the naked eye, but were seen microscopically to consist largely of small bacillary cords of average length 4–6 μ. These would appear in viable counts as single colonies, so that the count had to be regarded as an estimate of the number of bacillary units present in the culture, and fell far short of the total cell population. No means were found whereby the bacillary cords could be effectively disrupted.

(2) *Inoculation of mice.* Male white mice (Swiss albino no. 1), weighing 18–20 g., were arranged in groups of 15–20, evenly matched in weight. In most of the experiments, the mice were anaesthetized lightly with ether, and 0·05 ml. of culture injected intracerebrally. In some experiments, 0·05–0·2 ml. was injected intravenously. The methods used in balancing and maintaining such experimental groups have been detailed by Martin & Stewart (1950).

(3) *Assessment of results.* Cages were inspected daily and deaths recorded. All mice were examined post-mortem for the presence and severity of specific cerebral or pulmonary lesions, the latter being graded according to a system previously described (Stewart, 1950). A note was made of the mean time taken for the first half of the group to die with specific and extensive cerebral and/or pulmonary lesions (mean 50 % mortality time). After a period of 40–60 days, all surviving mice were killed and the lesions assessed post-mortem. The presence of bacilli was checked by smear or imprint preparations, and qualitative differences in the lesions by histological preparations.

RESULTS

Experiments with Mycobacterium tuberculosis *H* 37 *Rv*

Effect of route of inoculation. There was no significant difference in survival time between mice infected intracerebrally and mice infected intravenously with doses of 300,000–600,000 bacillary units. At higher doses (600,000–1,200,000 units) the mice proved significantly more susceptible to inoculation by the intracerebral route (Table 1). Within this dose-range, however, only a proportion of the mice in any one experimental group showed this increased susceptibility; such mice

Table 1. *Effect of various doses of* Mycobacterium tuberculosis (*H* 37 *Rv*) *inoculated intracerebrally and intravenously into groups of* 15–20 *Swiss albino mice*

Approximate inoculum (bacillary units in millions)	Route of inoculation	Mean mortality times of group in days		Percentage of group dead at (days)	
		50 %	100 %		
0·3	Intracerebral	42·0	—	30	(61)
0·3	Intravenous	46·0	—	20	(61)
0·6	Intracerebral	32·0	—	34	(47)
0·6	Intravenous	36·6	—	28	(47)
1·2	Intracerebral	15·8	>28·0	87	(49)
1·2	Intravenous	21·7	>28·0	92	(49)
2·4	Intracerebral	11·9	28·0	100	(35)
2·4	Intravenous	20·0	25·5	92	(35)
4·8	Intracerebral	6·0	12·1	100	(29)
4·8	Intravenous	19·8	24·0	100	(28)

died with cerebral lesions 8–20 days after inoculation, while the remainder of the group died with pulmonary lesions after the 20th day, as with mice infected intravenously. Inocula greater than 1,200,000 units given intracerebrally caused a further increase in the number of mice dying before the 20th day, but caused no appreciable further shortening of survival time in mice infected intravenously. In these experiments the inoculum-range 600,000–1,200,000 bacillary units therefore represents a threshold at which mice infected intracerebrally begin to manifest their maximum susceptibility to tubercle bacilli.

Effect of exact size of inoculum in mice infected intracerebrally. Intracerebral inoculation of 5000–10,000 units caused no appreciable mortality, though small proliferative lesions could be detected in the lungs between the 30th and 60th days of infection. Inocula of 20,000–300,000 units killed about half the mice with specific lung lesions, proliferative in character, within 60–70 days. Larger inocula (400,000–4,800,000 units) caused a progressive increase in mortality, 100 % mortality being attained with 2,400,000 units within 35 days.

When the mean 50 % mortality time was plotted against the number of bacillary units injected, a linear relationship was obtained, with a highly significant degree of correlation within the inoculum range 400,000–1,320,000 units (Fig. 1 and Table 2). With inocula of less than 400,000 units this linear relationship was lost,

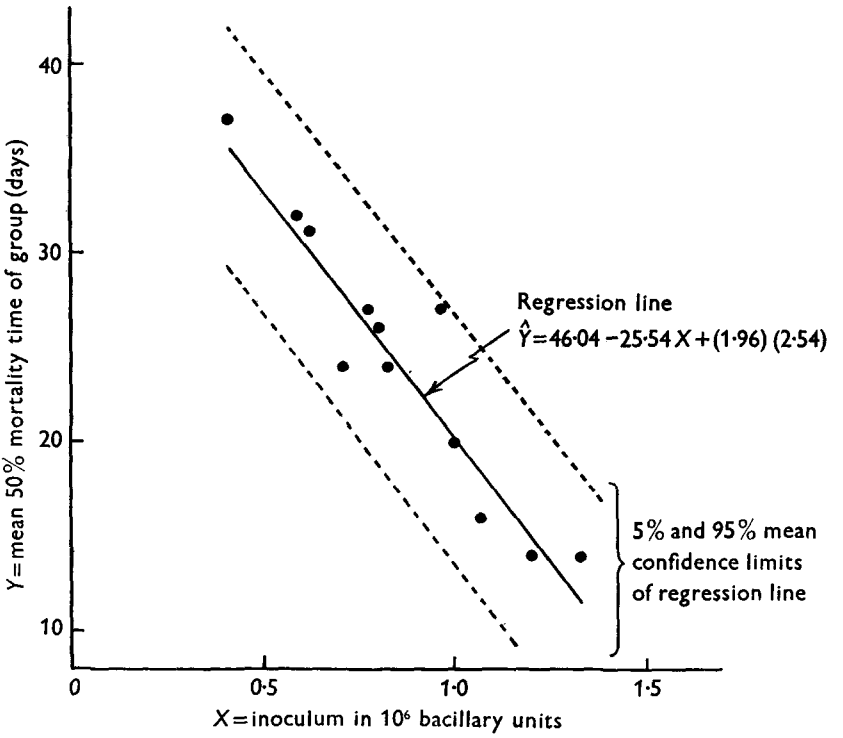

Fig. 1. Effect of *M. tuberculosis* H 37 Rv in mice infected intracerebrally. Regression of mean 50 % mortality time of group upon inoculum. (14th–16th weeks of serial subculture of organism.)

Table 2. *Mean 50 % mortality times of groups of mice inoculated intracerebrally with various doses of* Mycobacterium tuberculosis *(H 37 Rv)*

Inoculum in 10^6 bacillary units X	Mean 50 % mortality time of group Y	Regression estimate of Y $\hat{Y}$
0·400	37	35·58
0·580	32	30·90
0·600	31	30·38
0·700	24	27·78
0·760	27	26·22
0·800	26	25·18
0·820	24	24·66
0·960	27	21·02
1·000	20	19·98
1·060	16	18·42
1·200	14	14·78
1·320	14	11·66

Regression coefficient = 0·255. Regression equation $\hat{Y} = 46{\cdot}04 - 25{\cdot}54X$. $s = 2{\cdot}54$. Limits of $\hat{Y}$ are $\pm(1{\cdot}96)(2{\cdot}54)$ at $P = 0{\cdot}05$.

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though, at the other end of the range, regression remained linear with doses up to 5,430,000 units.

The reliability of the apparent dose-response relationship indicated by these findings depends upon the accuracy of the method for counting the number of bacillary units injected. Under ideal conditions of mixing the bacterial suspensions, and with a constant drop size, the number of organisms in a series of replicate drops should follow a Poisson distribution. A test for departures from these conditions is provided by the index, given by

$$\chi^2 = \frac{S(x-\bar{x})^2}{\bar{x}},$$

where x is the count in each of a series of replicates, $\bar{x}$ is the mean count and the symbol S denotes summation over the n counts. If the counts follow the Poisson distribution, this index follows approximately the χ^2 distribution on $n-1$ degrees of freedom; if $n=6$, as in the technique here, χ^2 would be less than 1·145 only 5 % of the time and greater than 11·070 only 5 % of the time. The results given in Fig. 2 show that, out of 32 series of 6 replicates, the index exceeded the upper

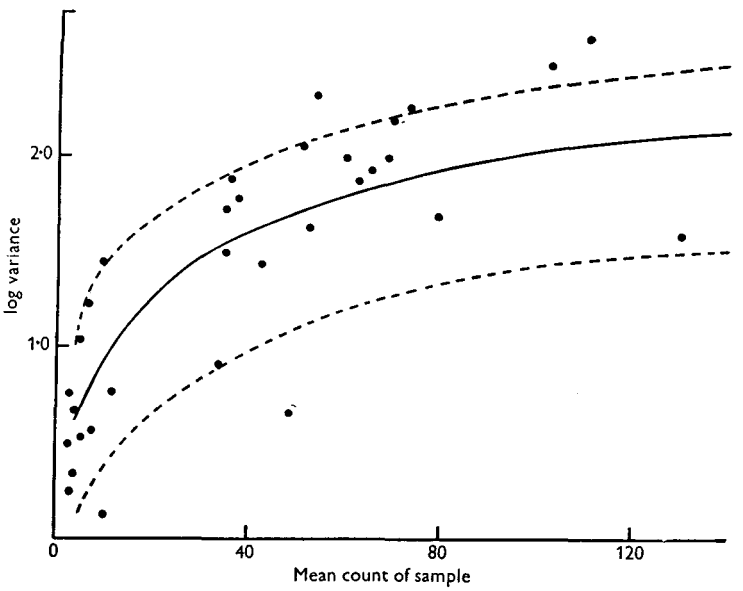

Fig. 2. Mean values and variance of 32 sets of colony counts at dilution 10^{-4}. Each point represents six samples, and the lines show the expected mean, 95 and 5 % levels on the basis of a Poisson distribution (*M. tuberculosis* H 37 Rv).

5 % limit only 5 times, and fell below the lower 5 % limit only twice. The expected frequency for each of these events is 5 % of 32, i.e. 1·6. These results therefore indicate a slight tendency for replicate counts to show more variability than would be expected under ideal conditions; the excess variability is, however, proportionately small.

In each experiment the dose was estimated from at least two sets of six counts, and should usually be accurate to within 10 %. This error in the dose makes

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a proportionately small contribution to the scatter of the points about the dose-mortality regression line (Fig. 1), and it was considered justifiable to follow the usual procedure in regression analysis, ignoring this error in the independent variable.

The regression line in Fig. 1 refers of course to results obtained with one strain of *M. tuberculosis* (H 37 Rv). If, for the purpose of the present investigation, we regard this strain as a standard of virulence, and suppose that its regression line is known exactly, then the relative virulence of a test strain may be estimated as

$$K = \frac{x'_1 x_1 + x'_2 x_2 + \ldots + x'_r x_r}{x_1^2 + x_2^2 + \ldots + x_r^2},$$

where x'_1 is the dose of the standard giving the same response as a dose x_1 of the test strain, etc. The doses $x'_1 \ldots x'_r$ are read from the standard regression line. K represents, in fact, the weighted average of the individual estimates of virulence x'_1/x_1, x'_2/x_2, etc., more weight being given to the high doses within the threshold inoculum-range, where these estimates are expected to be more accurate. The standard error of K is

$$SE(K) = \frac{s}{b\sqrt{(x_1^2 + x_2^2 + \ldots + x_r^2)}},$$

where b is the slope of the standard regression line of response on dose, and s is the residual standard deviation of the responses about this line. The 5 and 95 % mean confidence limits of the line are given by ordinates of $1{\cdot}96s$ from the line, in the direction of the time-axis.

If we assume that H 37 Rv represents, arbitrarily, 100 % virulence for the tubercle bacillus, then individual estimates x'_1/x_1 provide values which may be called *virulence ratios* of test strains, and K gives a more accurate estimate where two or more points are available for the test strain. In the present investigation, the virulence ratio and its standard error are expressed as percentage values of the (100 %) virulence of H 37 Rv.

Pathological changes in mice infected intracerebrally. When tubercle bacilli (H 37 Rv) were injected into the brain, sections or imprint preparations taken from mice killed 2–4 hr. later showed many single and clumped acid-fast bacilli scattered widely throughout the brain, and isolated organisms in the lung, spleen and liver. At this stage, the bacilli were large, thick and uniformly stained. Very few showed beading and the great majority were extracellular. The brain showed some haemorrhage and slight infiltration with polymorphonuclear leucocytes. After 24 hr. the bacilli in the brain were still widespread but many were present as clumps of short, small, deeply and evenly stained acid-fast forms. In the next 24 hr. the bacilli decreased in numbers, were mainly beaded and intracellular, and the distribution was localized into small foci, with accompanying gliosis. At this stage organisms were very difficult to find in the spleen or lungs. At 4 days the organisms in the brain were well localized, not numerous and almost entirely intracellular. Between the 8th and 20th days the bacilli increased sharply in number and an intense meningo-encephalitis developed. The meninges were hyperaemic. Many of the cortical vessels also showed marked congestion, and cuffs of mononuclear cells and lymphocytes. Areas of necrosis and dense cellular

foci were present in both hemispheres. Acid-fast bacilli were very numerous, often filling the cytoplasm of the large mononuclears which were predominant in the cellular areas. Many acid-fast bacilli were present as cords, lying extracellularly, similar to those observed in cultures in the Dubos liquid medium. In some instances, a dense mass of bacilli grew at the site of inoculation and, in general, the pathological changes were most marked in the inoculated hemisphere. Mice dying between the 10th and 20th days, however, showed widespread inflammatory changes and bacterial multiplication throughout the brain.

Changes of this type were observed in the brains of mice killed or dying up to about the 30th day of infection and subsided to some extent thereafter. The exact point at which the changes were maximal depended upon the dose of bacilli inoculated and was reflected in the early (8–20 days) 'cerebral' deaths of mice inoculated with 600,000–1,200,000 bacillary units. Lesions developed with smaller inocula, but were seldom lethal.

Pulmonary lesions developed rapidly after the 20th day of infection. Mice dying at 20–30 days showed extensive necrotic lesions containing dense masses of acid-fast bacilli; those dying later showed lesions composed of proliferating cells, containing fewer bacilli. Again, this difference in the types of lesion depended upon the dose injected. The critical inoculum range of H 37 Rv shown in Fig. 1 (400,000–1,320,000 units) always produced a high proportion of necrotic lesions. Splenomegaly, and minor lesions in other viscera, were observed after the 20th day, the splenomegaly being most marked in those mice, infected with lower doses, which survived longest.

Behaviour of H 37 *Rv in different strains of mice.* In one experiment equal inocula of bacilli were injected intracerebrally into balanced groups of young mice belonging to three homogeneous strains.* Brown mice of the ABC strain proved more susceptible, and C 57 black mice more resistant to the infection than the Swiss albino mice used routinely in these experiments (Table 3). The higher susceptibility of the ABC mice was associated with extensive cerebral, pulmonary and pericardial lesions, appearing in that order.

Table 3. *Effect of equal intracerebral inocula of* Mycobacterium tuberculosis *H* 37 *Rv in mice of different strains*

Strain of mice	No. of mice	Mean 50% mortality time (days)	Percentage dead at 20 days	Percentage dead at 40 days
Swiss no. 1 (albino)	25	23·0	12	76
ABC (brown)	22	15·4	40	77
C 57 (black)	26	27·1	8	52

Stability of virulence of H 37 *Rv.* Throughout the period of these experiments H 37 Rv was maintained in the same way, i.e. by serial transfers, at intervals of 2–3 weeks, on Löwenstein-Jensen medium. For each successive experiment subcultures from the current serial transfer were made in liquid synthetic medium

* ABC and C 57 mice were kindly supplied by Prof. A. Haddow from stocks bred at the Chester Beatty Institute, Royal Cancer Hospital, S.W. 3.

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and adjusted to an approximately standard inoculum as described under 'Methods'.

The results obtained over 41 successive weeks of comparable experiments with this serial strain of H 37 Rv are shown in Fig. 3. Assuming the inoculum in each experiment to be the same, we find variation in response to be comparatively small after the 18th week of serial subculture. There is a difference, however, between the mean response obtained at 9–18 weeks and that obtained thereafter. This difference, divided by its standard error on 19 degrees of freedom, gives a t value of 2·89 which is significant ($P = < 0{\cdot}01$). A slight decline in virulence therefore occurred between the 9th and 18th weeks of serial subculture.

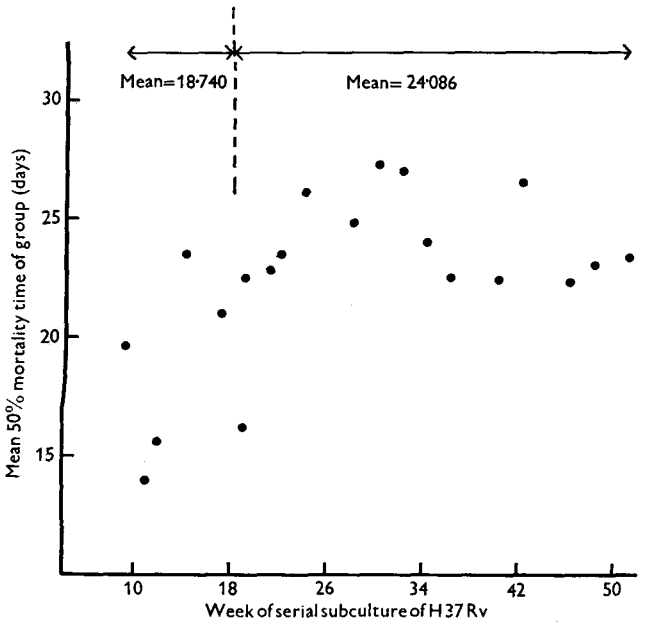

Fig. 3. Mean 50 % mortality time of groups of mice infected intracerebrally with approximately equal inocula of *M. tuberculosis* H 37 Rv over 42 weeks of serial subculture. t test:

$$t = \frac{\text{difference of means}}{\text{standard error of difference}} = -\frac{5{\cdot}346}{1{\cdot}849} = 2{\cdot}89.$$

The points forming the standard dose-response regression line of H 37 Rv (Fig. 1) were obtained in experiments at the 14th and 16th weeks of serial subculture. This is within the period during which decline in virulence became evident. A check was therefore made of the responses obtained with different doses of the standard strain at the 28th–32nd weeks (Fig. 4). The 'shadow' points thus obtained, though fewer in number, were distributed within the ordinary confidence limits of the standard regression line and their corrected virulence ratio showed no significant departure from the original value. This indicates that the responses obtained with the standard serial strain of H 37 Rv were stable between the 14th and 32nd weeks, and that the decline in virulence referred to above occurred suddenly at about the 12th week. Unfortunately, the experimental

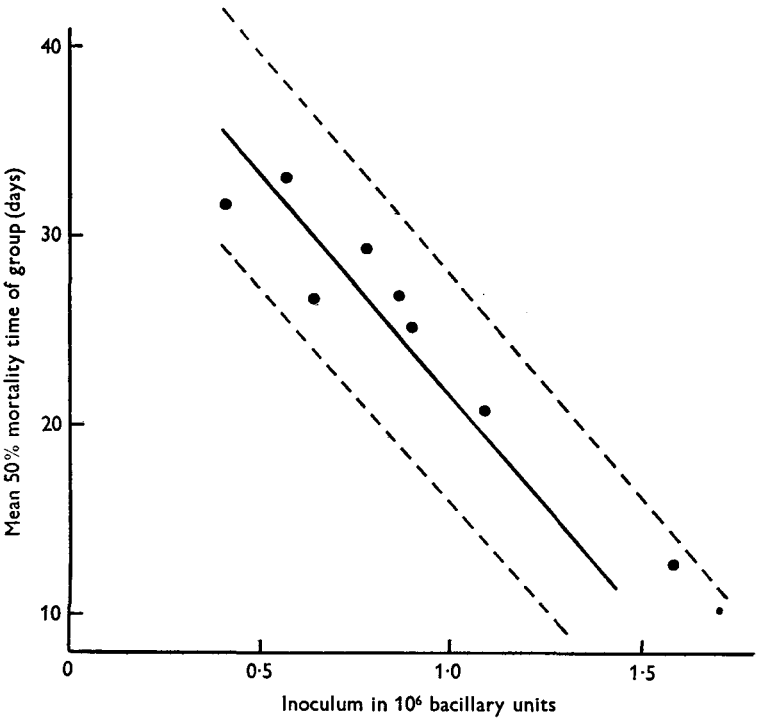

Fig. 4. Effect of *M. tuberculosis* H 37 Rv in mice infected intracerebrally at 28th–32nd weeks of serial subculture of organism. Comparison with original responses. (The lines indicate the original regression of H 37 Rv, with 5 and 95 % confidence limits.)

Table 4. *Effect of passage on the virulence of* Mycobacterium tuberculosis *H 37 Rv*

Passage			Mouse test		Corrected virulence ratio (% of standard)	Standard error (%)
Animal	Tissue	Duration (days)	Inoculum in 10^6 bacillary units	DT_{50}* (days)		
Mouse	Lung	49	0·840	27·5	91	6·0
	Lung	49	1·420	11·2		
	Brain	49	0·860	28·5	93	6·5
	Brain	49	1·300	11·8		
Rabbit	Lung	28	0·540	15·9	162	13·7
	Lung	28	0·494	32·4		
	Lung	135	0·350	21·3	175	13·0
	Lung	135	0·700	18·5		
	Kidney	28	1·220	20·0	(One point 82)	—
	Kidney	135	0·425	28·9	119	9·0
	Kidney	135	1·000	16·3		
Mouse	Lung	51	0·386	17·8	5-day cultures. Standard not applicable.	
Guinea-pig	Spleen	180	0·372	22·3		
No passage	—	—	0·107	26·3		
No passage	—	—	0·214	15·7		

* Mean 50 % mortality time of group.

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conditions between the 1st and 9th weeks of serial subculture were slightly different from those established and maintained thereafter, so that results obtained at 1–9 weeks cannot be used to investigate more precisely the manner in which this decline in virulence occurred.

The results quoted below with other strains of *M. tuberculosis* and modified strains of H 37 Rv refer only to experiments performed between the 14th and 51st weeks of serial subculture when the virulence of the standard remained stable.

Effect of passage. Mice and rabbits were infected with H 37 Rv during the 22nd–42nd weeks of serial subculture, when the virulence was stable. After various periods, the organism was re-isolated from lesions in the lung, kidney and, in mice, the brain. Subcultures pooled from these primary re-isolations were prepared, adjusted by the usual technique, and tested in mice against the serial strain of H 37 Rv which was, at about this time, rechecked for dose-response regression (Fig. 4). The results (Table 4) showed that passage in the brain or lung of the mouse caused no significant alteration in virulence. Passage in the lung of the rabbit enhanced the virulence ratio by about 50 %. This represented a significant increase in the mean virulence of H 37 Rv over the period of testing, but it was probably not significantly different from the virulence displayed by the organism at the 9th and 12th weeks of serial subculture (Fig. 3).

These experiments were repeated, using 5-day subcultures of organisms which had been re-isolated from lungs of mice and the spleen of a guinea-pig. No significant alteration in virulence was found (Table 4).

Table 5. *Virulence of tubercle bacilli from cultures of different ages in synthetic liquid media*

Strain of *M. tuberculosis*	Age of culture (days)	Inoculum in 10^6 bacillary units	Mean 50 % mortality time	Virulence ratio (%)
H 37 Rv	3	0·200	20·0	500
	4	0·109	22·3	825
	7	0·285	18·3	375
	12	0·432	27·5	164
	12	0·864	23·1	101
	25	0·281	>43·0	>50
	34	1·470	27·0	49
H 37 Ra	3	0·240	>72·0	
	12	0·330	>47·0	Very low
	12	0·700	>47·0	
BCG	3	1·080	>40·0	Very low
	12	1·280	>40·0	

Effect of age of culture. In most of the experiments, cultures of 11–12 days' growth in synthetic liquid medium were used, mainly for the reason that good growth occurred by that time in the majority of cultures examined, but partly because a fall in virulence was sometimes found if the cultures were older. When the present investigation was nearly completed, a paper by Bloch (1950) drew attention to the fact that younger cultures might be more virulent than those

which had attained optimal growth. Bloch's claim was therefore tested with cultures of 3, 4 and 7 days' growth, concentrated by centrifugation at 3000 r.p.m. and adjusted to standard turbidity. Even when equal turbidity was attained, however, clumping and cord-formation were more marked in the younger cultures than in the standard 11 to 12-day cultures, and counts registered lower numbers of bacillary units (Table 5).

When young cultures of H 37 Rv, prepared in this way, were injected into mice, a considerable increase in virulence was evident. The virulence ratio shows this increase as four- to eight-fold, but these ratios are probably unduly large owing to the apparent reduction of the calculated inoculum caused by the clumping.

Table 6. *Effect of type cultures of* Mycobacteria *in groups of* 15–20 *mice infected intracerebrally*

Species	Strain	Type no.	Inoculum in 10^6 bacillary units	Mean 50% mortality time	Deaths 0–25 days	Deaths 26–50 days
M. tuberculosis	H 37 Ra	NCTC 7417	0·330	> 50	—	—
	H 37 Ra	NCTC 7417	0·624	> 50	—	—
	H 37 Ra	NCTC 7417	3·000	> 50	—	1
	BCG	NCTC 5692	0·475	> 42	—	2
	BCG	NCTC 5692	1·088	> 40	—	2
	BCG	Copenhagen	1·200	> 40	—	1
	R 1	NCTC 7358	0·620	> 50	—	1
	R 1	NCTC 7358	1·044	> 45	—	2
	R 1	NCTC 7358	1·700	31·5	1	7
	M	—	0·530	> 50	—	6
	M	—	1·392	41·5	—	9
M. phlei	Type	NCTC 525	0·442	> 50	—	2
Vole bacillus	Type	NCTC 5676	0·984	> 48	2	2
	Type	NCTC 5676	1·800	18·8	5	11

Even if some allowance is made for this, however, there is still clear evidence of an increase in the virulence of the younger cells. Deaths within 8–20 days with extensive cerebral lesions were more frequent: severe, necrotic, pulmonary and sometimes myocardial lesions were observed, comparable to those seen in the susceptible ABC strain of mice described above. The acute 'Yersin' type of tuberculosis described by Bloch was not encountered.

Older cultures (25 and 34 days) showed a significant decline in virulence. There was no additional clumping error at this stage of growth and the counts registered were strictly comparable to those of the standard 11 to 12-day cultures.

Young cultures of attenuated strains (BCG and H 37 Ra) were also tested similarly, but failed to show any gain in virulence.

Experiments with other standard strains of Mycobacterium tuberculosis *and other mycobacteria* (Table 6)

With two exceptions, the strains referred to below were obtained from the National Collection of Type Cultures. The strains were maintained and prepared for testing in the same way as H 37 Rv.

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M. tuberculosis H 37 Ra (Ra). This organism is well known as a variant of H 37 Rv and is almost devoid of virulence for the guinea-pig. Experiments in mice (Table 6) showed that relatively enormous doses were tolerated, even by the intracerebral route. Some multiplication of the bacilli occurred in the tissues of the mouse, and microscopic proliferative lesions could be found in the lungs after 50 days. Even the largest doses, however, killed few mice within this time. When grown in liquid or solid Tween-albumin synthetic media, Ra failed to form the firm cords characteristic of H 37 Rv.

Table 7. *Effect of type strains of* Mycobacterium tuberculosis *in guinea-pigs (groups of* 4) *infected subcutaneously*

Strain	Dose (mg. moist weight)	Survival-time (days)	Lesions
H 37 Rv	0·01	60–150	Generalized extensive caseous lesions
H 37 Rv	0·05	25–96	
H 37 Rv	0·1	40–80	
H 37 Ra	0·1	> 180	Small proliferative lesions in glands and lungs
BCG	2·0	> 180	
M	0·1	> 180	Generalized caseous lesions with marked fibrosis
M	1·0	64–130	
R 1	1·0	> 180	Glandular lesions

BCG. Two strains of this organism were tested, one being the strain supplied by the State Serum Institute, Copenhagen, for use as a vaccine, and the other the strain maintained by the National Collection of Type Cultures. Both strains grew rapidly on Löwenstein-Jensen media and formed thick, rather loose cords in liquid or solid Tween-albumin media. The NCTC strain grew quickly and luxuriantly in liquid media, attaining full turbidity in 3–5 days.

Both strains of BCG proved slightly more virulent to mice than Ra, in that occasional deaths occurred within 50 days and proliferative pulmonary lesions, usually microscopic in size, could be more easily found.

R 1 ('American avirulent'). This organism formed typical, though relatively scanty cords on Tween-albumin media. The virulence to mice was rather higher than BCG, and macroscopic lesions, entirely cellular in pattern and containing moderate numbers of bacilli, formed slowly in the lungs. As with BCG, a few deaths occurred between 25 and 50 days, probably due to a combination of late cerebral and pulmonary lesions.

M. tuberculosis, strain M, was isolated in 1947 from the lung of a monkey suffering from natural tuberculosis. The organism grew well on Tween-albumin media, and formed typical cords. Since first isolated, this strain has been consistently sub-virulent to guinea-pigs. In mice, 50 % mortality was reached in 50 days and macroscopic proliferative lesions were present in all survivors. No early cerebral deaths occurred. This strain was therefore intermediate in virulence between R 1 and H 37 Rv.

M. phlei and the vole acid-fast bacillus were also tested. These strains grew quickly and luxuriantly in Tween-albumin media, forming no cords. In mice

M. phlei was virtually avirulent though perhaps not devoid of infectivity, for a few small cellular foci containing acid-fast bacilli were found. The vole acid-fast bacillus multiplied rapidly in all tissues and caused an appreciable mortality. The 'lesion', however, consisted mainly of masses of bacteria, with only a minimal tissue reaction.

BCG and Ra were tested in cultures of different ages, young and old, without revealing any definite differences in virulence.

The strains of tubercle bacilli described above were also tested in guinea-pigs (Table 7). H 37 Rv caused a generalized fatal disease which was not necessarily shortened by an increase in dose. Ra, R 1 and BCG caused only minimal lesions and permitted survival for 6 months at least. Strain M caused extensive lesions but the lethal dose was high.

DISCUSSION

Before the virulence of different strains of tubercle bacilli can be assessed, three major technical conditions must be met:

(*a*) The method of testing must be such that, either exactly equal inocula of the different strains are used, or a means must be found of applying a reliable correction for any differences in inocula.

(*b*) A fixed standard of virulence must be established under reproducible experimental conditions.

(*c*) The standard must be based upon inocula which give a threshold response.

These conditions are not of course peculiar to work with the tubercle bacillus, but they are certainly of unusual importance in the case of this organism. In the first place, inocula are not easily standardized, especially where various strains with different growth characteristics are being compared with one another. Adjustment by moist or dry weight does not reflect accurately the numbers of viable organisms in the inocula (Wilson & Schwabacher, 1937; Glover, 1946). Turbidimetric standardization carries this same hazard, together with additional optical factors which demand that the bacterial cells be thoroughly washed and evenly dispersed. As a solitary criterion, viable counts carry, in the case of tubercle bacilli, the twin difficulties of clumping and slow growth.

In the experiments reported here, the inocula were adjusted essentially by making approximately equal cell-suspensions from cultures at the height of their growth, and checking differences caused by clumping or dead cells by a series of viable counts. Differences in response associated with the inevitable, though relatively fine, differences in the exact inocula were corrected by using a sliding-scale standard. This was provided, within statistically defined limits, by the regression line of response upon dose of the strain H 37 Rv which behaved, within the greater part of the period of testing, as a fixed standard of virulence. The regression line of this strain covered the inoculum-range at which, for biological reasons, threshold responses could be recognized.

Given this fixed standard, the relative virulence of test-strains over an inoculum range of at least 400,000–1,300,000 bacillary units could be assessed quantitatively and expressed as a (percentage) virulence-ratio of the (100 %) standard H 37 Rv.

Further accuracy was attained by weighting the average of two or more individual estimates, at different inocula, in terms of the more accurate estimate which could be expected with the higher inocula.

This method was based upon the assumption that the standard response-dose line was known exactly. In fact, the original line (Fig. 1) was estimated from only 12 points; this discrepancy was relatively unimportant, however, since it was intended to use for comparison only two points, as a rule, for each test strain. Consequently, most of the sampling fluctuation in a (weighted) estimate of virulence would be due to sampling fluctuation in the test results. Furthermore, the validity of the original response-dose line was strengthened by the fact that the second regression estimate, made midway through the tests, showed no significant departure (Fig. 4).

During most of the period in which our experiments were made, the mouse-virulence of H 37 Rv was remarkably stable (Fig. 3). If identical conditions could be maintained in subsequent experiments, the responses obtained with H 37 Rv or a similar stable strain could be adopted as a permanent standard of virulence, admittedly of an arbitrary nature. Many factors, however, must necessarily interplay in determining such experimental conditions: strain, age, sex, weight and diet of the mice, and variations in culture media, to mention but a few. The standard must therefore be constantly employed, not only to register changes in the experimental conditions, assuming the standard itself to be stable, but also to check the latter assumption. The slight decline in virulence of H 37 Rv noted after the 12th week of serial subculture (Fig. 3) showed that alterations could occur quickly, so that a constant check was required.

Quantitative assessments of the virulence of tubercle bacilli are more illuminating when they are properly understood in relation to certain qualitative variations in the types of lesions caused by different strains of different sized inocula of the organism. Virulent strains, of which H 37 Rv is a representative example (Stewart, 1951), showed three definite pathological characteristics at threshold inocula of 600,000–1,300,000 bacillary units. These characteristics were: (1) the development of cerebral lesions, 25–50 % fatal, within 8–20 days; (2) large necrotic lesions in the lungs at 20–30 days; (3) intense multiplication of the bacilli in the lungs.

In contrast, strains of very low virulence, such as H 37 Ra, R 1 and BCG caused only minute cellular lesions, no early cerebral lesions, and multiplied very slowly. Intermediate strains, of which strain M was an example, caused large but essentially proliferative cellular lesions in the lungs, with a curious foam cell reaction in the alveoli (Stewart, 1950), and multiplied relatively slowly, largely intracellularly.

These pathological differences between the strains were absolute only in relation to certain defined inocula. It has already been shown (Stewart, 1950) that a large dose of moderately virulent bacilli may produce a response very similar to that caused by a small dose of virulent bacilli. This overlap, however, does not extend as far as strains of very low virulence, such as H 37 Ra.

In the foregoing part of this discussion we have stressed the conditions under

which H 37 Rv was stable in virulence. The work of Bloch (1950), which was confirmed to some extent in our experiments, emphasizes further the importance of defining the experimental conditions employed. Young cells were more virulent than cells drawn from cultures at the height of growth, and older cells became more attenuated. We cannot from these studies altogether confirm Bloch's observation that young cells produced an acute, wholly different form of tuberculous lesion; our finding was that the evolution of disease was accelerated in a manner comparable to that observed in the highly susceptible, cancer strain of ABC mice (Table 3). Also, inocula prepared from 3- to 7-day cultures require to be concentrated considerably, and represent a sample selected at a time when young, intensely corded cells form the major part of the total cell-population. If we assume, as Bloch suggests, that such cells are the essential virulent components of a culture, then a sample taken at 3–7 days will produce a response weighted by their increased presence.

Passage in the mouse and guinea-pig failed to alter the virulence of H 37 Rv. Passage in the lung of the rabbit increased the virulence of this strain at the time, but may not have increased it beyond a level originally possessed. The more highly virulent, younger cells were not further exalted by passage.

In the foregoing discussion, the term virulence has been used to denote the degree of pathogenicity shown by a given organism in a given host. On this basis, a standard of virulence can be established, but it is by definition arbitrary and refers to a host parasite relationship which provides only one index of the potential pathogenicity of the organism.

SUMMARY

1. Mice infected intracerebrally with *M. tuberculosis* H 37 Rv in doses of 400,000–1,300,000 bacillary units gave a graduated series of responses whose regression provided an arbitrary 100 % standard of mouse virulence for human tubercle bacilli.

2. Under certain experimental conditions this standard remained stable and its confidence limits could be defined.

3. The relative virulence of other strains of tubercle bacilli could be assessed quantitatively in relation to this standard, and expressed as a (percentage) virulence ratio.

4. 100 % virulence was defined as that of a strain showing a threshold of response of 600,000–1,300,000 bacillary units, lethal cerebral lesions at 8–20 days or necrotic pulmonary lesions at 20–30 days, and a mean 50 % mortality time of less than 36 days.

5. Qualitative and quantitative responses characteristic of intermediate and very low virulence were also defined.

6. Higher proportions of virulent cells were obtained from 3 to 7 days than from older cultures in Tween-albumin liquid media, but inocula were more suitably standardized by obtaining cells from 11 to 12-day cultures.

7. Passage in the mouse and guinea-pig failed to alter the virulence of H 37 Rv. A slight increase was effected by passage in the lung of the rabbit.

8. The technique employed was a measure essentially of the ability of the organism to multiply in, and damage, resistant tissues.

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